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Short Communication

The effects of dog appeasing pheromone spray upon canine vocalizations and stress related behaviors in a rescue shelter

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Abstract

Exposure to dog appeasing pheromones (DAP) has been suggested to reduce stress related behaviors in dogs; however, the effects of DAP administered using a portable, rapid use spray has not received as much attention as the plug-in format. The aim of the present study was to determine whether DAP spray reduced stress related behaviors in rescue shelter dogs *(Canis familiaris)*. Barking intensity, frequency of barking and stress related behaviors in the presence of a stressor were recorded using a repeated measures design with and without the use of spray pheromones. The mean barking intensity was reduced in dogs exposed to DAP spray although no significant difference in the frequency of barking or occurrence of stress related behaviors was found. This change in barking behavior is difficult to interpret as being beneficial to dog welfare, due to the lack of support from a reduction in the other stress indicators. Further research is needed which utilizes both a longer time period of DAP exposure and behavioral observation to understand any effects of DAP on dogs' behavior. A larger sample size, alongside use of different stressors and physiological stress indicators, should also be considered.

Keywords: Dog Appeasing Pheromones; DAP Spray; Vocalizations; Dog Behavior

Introduction:

Each year large numbers of domestic dogs *(Canis familiaris*) are relinquished to animal rescue shelters. In 2009 approximately 129,743 dogs entered UK welfare organisations (Clark et al., 2012). Dogs enter shelters for many reasons, as strays or unwanted pets, or due to being relinquished by their owners as a result of undesirable behavior (Fatjó et al., 2006). Many dogs fail to find new homes and temporary kennel accommodation often becomes longer term housing. Long term confinement in kennels can be stressful as a result of social isolation, spatial restriction and changes in routine (Beerda et al., 1999). Over time, these factors can contribute to chronic stress and subsequently compromised welfare in dogs (Beerda et al., 1999).

Dog appeasing pheromones (DAP) are reported to be a chemical synthetic analogue of the natural canine appeasing pheromone produced by a lactating bitch to reassure the puppy (Pageat, 1999). According to the manufacturer, DAP promote calm behaviors in both young and adult dogs (Adaptil, 2016). These products have been reported to calm dogs in stressful environments such as kennels (Tod et al., 2005) and veterinary practices (Mills et al., 2006). DAP can be administered using either a collar, spray or diffuser. The DAP collar or spray can be used rapidly in areas where a plug-in diffuser is not practical, for example outdoor kennels that lack a power supply to individual enclosures. As the spray is portable, it can be used in any new areas where a dog may be fearful (Mills et al., 2006). In contrast, although a plug-in diffuser allows for a continuous and longer lasting application of DAP in a larger environment (Levine et al., 2007), it takes time to heat up and diffuse into the surrounding environment and requires up to 24.00h to become fully effective (Adaptil, 2016). This means that any desired effects of the product may not be observed in dogs that enter the environment until after a delayed time period. Consequently, a spray formulation may be more useful in eliciting a more rapid effect on problem behaviors.

While some studies have suggested that DAP may have some application in reducing anxiety in dogs (e.g., Tod et al., 2005 and Mills et al., 2006), further investigation of the efficacy of DAP in reducing canine stress is warranted. When used in combination with desensitization and counterconditioning programmes, DAP administered using a diffuser has been reported to reduce problem behaviors such as hyperactivity, excessive vocalizations and separation anxiety in noise phobic dogs (Levine et al., 2007). It is worth noting that, because of study design, effects due to the behavioral modification programme and the pheromone could not be separated by Levine et al., (2007), so it's not possible to know which aspect of treatment produced a reduction in fearful behavior (Frank et al., 2010). In these types of studies, a reduction in fearful behavior cannot be solely or accurately attributed to DAP and any potential effects of a behavioral modification programme need to be considered.

Previous research has suggested that DAP administered using a diffuser reduces stress and fear related behaviors in dogs in both a shelter environment and veterinary practice (Tod et al., 2005; Mills et al., 2006). Shelter dogs exposed to DAP emitted from a diffuser exhibit barking of a lower decibel level and reduced frequency, which was purported to show reduced stress levels (Tod et al., 2005). It is important to note though that while a reduction in barking amplitude and frequency was reported in Tod et al., (2005), statistical methods were used which caused results to not always be directly comparable among treatment groups (Frank et al., 2010). Consequently, comparisons between any effect of DAP as opposed to the control could not be reliably made. It has also been reported that initial exposure to DAP is effective in reducing signs of anxiety but not overt aggression in dogs in the veterinary clinic environment (Mills et al., 2006). However, methodological limitations, including an inadequate randomization scheme and unclearly defined inclusion criteria, need to be considered when interpreting the results of Mills et al., (2006). Neither study reported treatment outcome, so it is also unclear how many participants failed to respond to the DAP treatment. True pheromones are known to control behavior, but previous studies utilizing DAP, a synthetic analogue, have methodological limitations which make it inherently difficult to determine any true effectiveness (Frank et al., 2010).

Studies incorporating portable DAP (e.g., impregnated collars) have been used in canine travel-related research and postulated to be effective, to some extent, by controlling sympathetic arousal (e.g., Estelles and Mills, 2006). Previous study of the efficacy of DAP has tended to focus upon administration via diffuser or collar and, to our knowledge, no previous studies have examined the behavioral responses of dogs to DAP spray in a shelter setting. Spray administration may be beneficial in rescue shelters because it allows immediate application of product in areas, such as meet-and-greet rooms, where individual dogs may be viewed at short notice by potential adopters. Spray application may also be useful beyond the shelter environment if adopted dogs encounter short-term stressors, such as new introductions to existing animals within the household. The aim of this study was to determine whether DAP spray reduced vocalization intensity and frequency of stress related behaviors in dogs housed in a rescue shelter upon exposure to a stressor.

Materials and Methods

Subjects and Study Site

Twenty five dogs, 16 males (14 neutered, 2 entire) and 9 females (8 spayed, 1 entire) aged between 5 months and 168 months (mean age: 41.64 months) were used in this study (Table 1). Thirteen of the dogs were purebred, with the remaining dogs being cross or mixed breeds. Twelve of the dogs were strays and thirteen of the dogs were relinquished to the shelter. Dogs were placed into either small n = 2 (< 10 kg), medium n = 15 (> 10 kg but below 25 kg) or large n = 8 (> 25 kg) weight categories (Kim et al., 2011). All dogs were in good general health and were housed at Worcestershire Animal Rescue Shelter, Worcestershire, UK. The study took place using either 1.5 x 2.7 m kennels or 2.7 x 5.6 m kennels. Larger dogs and dogs who the shelter deemed as displaying high levels of behavior indicative of stress were put in the larger kennels and therefore kennel size was unable to be controlled within this study. Kennels were situated in a row with a wire mesh fronted barrier. Dogs were housed individually and each kennel contained a bed, blanket and water bowl. Dogs were fed at 08:15h and again at 14:00h. A walkway located 10 meters away from the outside of the kennels was used to exercise the dogs on a daily basis (approximately twice a day), so the focal dogs in this study were used to the presence of other dogs walking in front of the kennels. Data were collected outside of normal walking times (10:00h – 16:00h) and public viewing times (11:00h – 15:00h) to avoid the influence of other dogs and also human presence on the focal dogs' behavior.

Procedure

A repeated measures design was used to assess the behavioral responses of the dogs to exposure to DAP spray. These behavioral measures were scored in the presence of a 'stressor'- a neutral dog personally owned by the researcher who was unfamiliar to all dogs, who was led past the kennels (approximately at a 1 m distance) during data collection to induce a behavioral response so any effects of DAP could be measured. Dogs that were not participating in the study were either shut inside the kennel block or in the isolation block, which was separated away from the main kennels.

Dogs were allocated to an order of conditions depending on when they arrived at the shelter, with longer resident dogs allocated first followed by new arrivals. The conditions were counterbalanced (without DAP/with DAP, n = 12, with DAP/without DAP, n = 13) to control for order effects. Dogs were divided in to ten smaller groups for ease of observation. Each group of dogs experienced the control condition (without DAP spray) and the exposure condition (with DAP) which occurred on consecutive days with observations repeated twice a day at 09.00 h and 17.00 h. In the DAP condition, two pumps of the 60ml DAP spray were applied to each of the four corners of the kennel 30 minutes prior to exposure to the stressor to assess the effect of the spray on barking intensity, frequency of barking and other stress related behaviors (Tod et al., 2005; Levine et al., 2007). The spray was applied when dogs were removed from the kennel to allow the pheromone to dissipate into the environment and to allow alcohol evaporation (Tod et al., 2005; Levine et al., 2007). There was no placebo treatment in this study, and researchers were not blinded to treatment.

Dogs remained in the same kennel throughout the experiment. Observations were conducted 30 minutes after application of the product (as per Graham et al., 2005) with behavioral observations starting with the appearance of the stressor dog at approximately 1 meter from the kennel and each observation lasting 10 seconds. Focal sampling was used to record the frequency of behaviors displayed by the dogs. Behaviors potentially associated with canine stress, including low body posture, licking lips, yawning, panting and vocalizations (Beerda et al., 1999; Tod et al., 2005) (Table 2), were recorded, as was the barking intensity. Mean barking intensity (dB) was recorded during each 10 second observation using a decibel meter (Max Measure, Universal Supplies Ltd), located 15 meters from the kennel block and centralised to the kennels' centre using a marker. The frequency of occurrence of other stress related behaviors were captured using video recorded behavioral observations (Go Pro Hero, Foxconn). The Go Pro Hero was hand held by the researcher, while walking the stressor dog past the focal dogs, and was set on 720p resolution, 60 frames per second and set in 'super view' mode to capture multiple dogs' behaviors at the same time. Dogs in each group were recorded at the same time to avoid repeated exposure to the stressor dog and therefore minimise habituation or sensitisation. Video footage was analysed at a later date and video files were renamed by the researcher prior to analysis to minimise observer bias.

Statistical Analysis

Decibel readings with and without the use of the DAP spray were recorded and summed to provide a mean dB reading per condition per group. The frequency of dogs displaying the behavior was summed providing an overall frequency count per dog per behavior. For auditory analysis, paired t-tests were performed to test for differences in the decibel level of dogs between the two conditions, with and without the DAP spray. Paired t-tests and Wilcoxon signed-rank tests were used to test whether there was a statistically significant difference in behavior with and without the use of pheromones. These tests were chosen according to whether the assumptions underlying parametric analysis were sufficiently met. All data were checked for normality using Kolmogorov-Smirnov tests. The significance level was set *a priori* at $p = 0.05$ and all statistical analysis was performed using SPSS (version 22, 2013).

Results

Exposure to DAP spray in the presence of a stressor resulted in no significant differences in the occurrence of stress related behaviors, however significant effects upon intensity of barking were found.

Barking Intensity

There was a significant difference in barking intensity when dogs were exposed to DAP spray ($t = 4.329$, df = 9, $P = 0.002$). The mean barking intensity was lower in the DAP spray condition as opposed to when dogs were not exposed to DAP (Table 3: DAP spray = 57.16 dB, no DAP spray = 63.64 dB).

Non-Significant Behavior

There were no significant differences in frequency of barking (t = 0.000, df = 24, P=1.000), paws on the fence (t = -1.633, n = 25, P= 0.102), low posture (t = -0.816, n = 25, P= 0.414) and lying down (t = -1.667, n = 25, P= 0.096) (Table 3). Where behaviors were exhibited at very low levels (mean occurrence < 1) they were omitted from analysis as statistical analyses are not robust at such low levels.

Discussion

The present study is the first, to our knowledge, to test the efficacy of DAP spray in reducing vocalization intensity and frequency of stress related behaviors in shelter dogs. No significant differences in stress related behaviors or barking frequency were found in this study, although small differences in mean barking intensity in the presence of a stressor were found in dogs that were exposed to DAP spray. Barking intensity was lower in the condition where dogs were exposed to DAP spray. It is difficult to conclude that the small reduction of 6.48dB in loudness in the DAP condition is clinically or biologically significant or beneficial for the dogs' welfare.Our results should be interpreted with caution when attempting to draw conclusions regarding DAP and shelter dog welfare.

Alternative explanations need to be considered. Rescue shelters can be a stressful environment for dogs due to psychological and physiological stressors (e.g., noise and both spatial and social restrictions) (Hubrecht, 1995; Tuber et al., 1999; Taylor and Mills, 2007). It is possible that the level of stress experienced in the shelter environment in this study, whether due to the stimulus of the stressor dog, or due to the kennel environment itself, may have been too great for DAP to have a marked effect on the dogs' behavior, if pheromonal analogue products produce only mild effects. Both social isolation and the inability to control the environment and behavioral opportunities have been suggested as stressful to dogs (Hubrecht, 1995; Tuber et al., 1999; Taylor and Mills, 2007). It is possible that the presence of the stressor dog walking past the kennels and the kennelled dogs not having the opportunity to interact with the individual or having the ability to control the interaction may have resulted in sufficiently high stress levels, that such products are not adequate redress. Similarly, the shelter environment may have been too stressful for such products to have a noticeable effect on the dogs' behavior. Further controlled, blinded studies considering the use of DAP in response to different stressors and in different situations would be useful to determine whether use of the product is warranted at all, or only indicated in restricted contexts.

In both conditions in our study, behavioral responses such as barking frequency, paws on fence, low posture and lying down remained unchanged. Future studies of DAP could combine behavioral indicators with non-invasive sampling of saliva to see whether there are any physiological changes relating to distress exhibited in rescue shelters, which are deemed as stressful environments for dogs (Hubrecht, 1995; Tuber et al., 1999; Taylor and Mills, 2007).

Behavioral responses have been found to vary substantially between individuals in a rescue shelter environment (Steven and Ledger, 2005) due to temperament (Jones and Gosling, 2005) and coping style (Steven and Ledger, 2005), which can be attributed to genetic factors such as breed and sex (Serpell and Hsu, 2005) and to environmental factors such as experience (Appleby et al., 2002), rearing environment (Harvey et al., 2016) and neuter status (Serpell and Hsu, 2005). Previous studies have reported large individual variations in behavior of kennelled dogs (Hubrecht, 1995 and Titulaer et al., 2013). The small sample size used in our study may have meant that behavioral variation was limited in the dogs observed. If the effects of pheromonal analog products are restricted to a range of behavioral presentations, these may not have been represented in a small sample size study.

Because of the lack of effect of DAP on the stress indicators assessed in this study, such treatment does not enhance welfare under these study conditions. However, novel, stimulating and unpredictable environments like shelters may facilitate barking (Tod et al., 2005). High noise levels caused by vocalisations can implicate welfare through potentially damaging dogs' hearing in shelter situations in a relatively short period of time (Scheifele et al., 2012). It is possible that some dogs in this study may have had altered hearing, given their exposure to barking dogs housed in the shelter, and that this potential outcome, which occurs with time, may have changed behavior. Kennelled dogs are regularly exposed to sound levels over 100dB and it has been reported that noise levels in excess of 100dB can damage dogs' hearing (Scheifele et al., 2012). Since only a low dB range (51-73 dB) was recorded in both conditions in the present study, welfare was unlikely to have been impacted through hearing loss. The effect size in this study was small, with only a 10.1% decrease in noise intensity found in the DAP condition. Such a small decrease of 6.48dB, which was still within the low dB range reported, is unlikely to have improved welfare by reducing the risk of hearing loss in this study. The low range of dB readings recorded may have been attributed to the location of the decibel meter, which was located 15 meters from the kennel block and may have been located too far away to record dB readings accurately. Future research could consider placing microphones centrally within the kennel and suspended from the ceiling so they are closer and within the hearing zone of the individuals (Scheifele et al., 2012).

As kennels are widely known to be noisy environments (e.g. Sales et al., 1997; Coppola et al., 2012; Scheifele et al., 2012) with noise levels regularly exceeding 100dB and often reaching 125dB (Sales et al., 1997), it may be more prudent for shelters to implement noise abatement measures instead of DAP and improve welfare through minimising the risk of hearing loss. Such measures could include absorptive surfaces to decrease reverberation and increased levels of sound insulation in kennels which may help reduce high sound levels (Sales et al., 1997).We studied the dogs only when no visitors were present and when no other manipulations (e.g., feeding) were ongoing. If a decrement in barking was shown to occur in a controlled study in the presence of DAP when others were present and/or more active manipulations occur, then pheromonal analogues may have application in rescue shelters if a reduction in barking is perceived as desirable by adopters.

There are a number of limitations to this study, such as the sample size, the use of only one type of stressor and lack of control for breed, age or residency duration effects upon barking intensity. Residency duration was confounded with order effects which may have impacted how longer resident dogs reacted to the stressor dog. There was also an assumption that the stressor dog acted the same way during each exposure, however the stressor dogs behavior was not measured. Measuring sound intensity in a kennel environment is also difficult due to sources of noise from other dogs and equipment therefore background noise and socially facilitated barking may have also confounded measurements of barking intensity. While these confounding variables are difficult to control, they should be considered when interpreting the results of this study. Additionally, location of dogs in kennels and weight versus kennel size were not able to be controlled and may have affected level of exposure. It is also possible that more rarely exhibited behaviors were missed due to the short recording period used in this study (Martin and Bateson, 2007). This study was neither blinded, nor had a placebo control, which would have allowed us to evaluate any effect of actually doing the study on outcome. Future research on any potential effects of pheromonal analogue products on shelter dogs should redress these limitations.

Conclusions

In summary, application of DAP spray was associated with a small reduction in barking intensity in shelter dogs upon exposure to a stressor in this open label, non-placebo controlled study. Our results should be interpreted with caution as a small reduction in dB level does not mean the results are clinically or behaviorally significant. Other behavioral indicators of stress were not observed to decrease in a statistically significant manner in a way that paralleled the reduction in bark volume. Dogs bark for a variety of reasons, and it’s beyond the scope of this study to assign attribution for the barking, given the experimental design.

Conflict of interest statement

The authors have no conflict of interests to declare. None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Ethical considerations

Approval for the study was not needed under the Animals Scientific Procedures Act 1986 or the European Union Directive 2010/63/European Union. The study abided by the guidelines of the Institutional Research Ethics Committee.

Authorship

The idea for the article was conceived by Christopher Hermiston and Sienna Taylor. The experiments were designed by Christopher Hermiston and Sienna Taylor. The experiments were performed by Christopher Hermiston. The data were analyzed by Christopher Hermiston. The article was written by all the authors.

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Table 2

Ethogram providing definition of behaviors sampled in the DAP spray study (adapted from Beerda et al., 1999 and Tod et al., 2005).

Behavior	Definition
Body posture and motor activity:	
Lying	Ventral/lateral lying on ground with all four legs resting and in contact with ground. Eyes may be open or closed.
Sitting	Hind quarters on ground with front two legs being used for support.
Paws on fence	Standing on two hind limbs supporting body other front legs against the fence.
Walking	Forward movement with legs resulting in shift of whole body to a new position in enclosure.
*Low posture	Head lower than shoulders, tail low, ears lowered.
Spinning	Rotating the body 360 degrees around.
Jumping	No limbs on the floor.
Vocalizations:	
*Bark	‘Rough’ sound often repeated in quick succession.
*Growl	Deep threatening rumble.
*Yelp	Sustained high pitched sound related to howling/ barking.
Displacement:	
*Yawn	Mouth opens wide for a period of a few seconds, then closes.
*Lick Lips	Tongue extends upwards to cover lips, before retracting into mouth.
*Pant	Mouth opens with tongue extended accompanied with rapid breathing and expansion/contraction of chest.

Escape Behavior:	
Exit rear	Standing on hind legs with front legs resting against exit
Wall bounce	Standing on hind legs with front legs rebounding off wall—usually repetitive
Bar pawing	Using paws to reach through mesh exit—in a digging motion
Exit stare	Dog's gaze focused on exit points.
Exploratory Behavior:	
Sniff	Air inhaled forcibly through nose.
Lick object	Tongue extends to touch object before retracting into mouth.
Nose/paw object	Use of paw/nose to manipulate object.

*Indicates stress related behaviors

Appended Table

Table 1: Demographics of population sample

Breed Composition	**Dog ID Number**	**Sex**	**Status**	**Approximate Age (Months)**	**Length of Residency in Shelter (Rounded up to nearest week)**
Labrador	F1	F	Neutered	85	3 weeks
English pitbull terrier	F2	F	Neutered	48	3 weeks
Chihuahua/Jack Russell	F3	F	Neutered	12	2 weeks
Collie	M1	M	Entire	5	3 weeks
Great Dane	M2	M	Neutered	12	4 weeks
Saluki, lurcher cross	M3	M	Entire	20	12 weeks
Great Dane	M4	M	Neutered	12	4 weeks
Lurcher	F4	F	Neutered	19	18 weeks
Labradoodle	F5	F	Neutered	30	9 weeks
Husky, collie cross	F6	F	Neutered	26	16 weeks
Trailhound	F7	F	Entire	88	12 weeks
Lurcher	M5	M	Neutered	53	5 weeks
Springer apaniel	M6	M	Neutered	40	1 week
Deerhound	M7	M	Neutered	11	1 week
Staffordshire bull terrier	M8	M	Neutered	16	1 week
Collie	M9	M	Neutered	168	1 week
Husky	M10	M	Neutered	18	1 week
Akita	M11	M	Neutered	38	1 week
Labrador	M12	M	Neutered	41	1 week
Lurcher	F8	F	Neutered	11	1 week
Trailhound	M13	M	Neutered	53	2 weeks
Trailhound	F9	F	Neutered	53	2 weeks
Lurcher	M14	M	Neutered	129	2 weeks
Collie	M15	M	Neutered	26	2 weeks
Staffordshire bull terrier	M16	M	Neutered	27	2 weeks

Table 3: Summary of raw behavior and decibel data

	Behavior Data								***Decibel Data (dB)**						
	N = 12 Without DAP				N = 12 With DAP					N = 12 Without DAP			N = 12 With DAP		
Dog ID	**Frequency of Barking**	**Frequency of Paws on Fence**	**Frequency of Lying**	**Frequency of Low Posture**	**Frequency of Barking**	**Frequency of Paws on Fence**	**Frequency of Lying**	**Frequency of Low Posture**	**Group Number**	**dB Reading AM**	**dB Reading PM**	**dB Reading Average**	**dB Reading AM**	**dB Reading PM**	**dB Reading Average**
F1	2	0	0	0	2	0	0	0	Group 1	60.66	60.15	60.41	57.96	53.23	55.60
F2	0	0	0	0	0	0	1	0							
F3	3	0	0	0	3	0	0	0							
M1	0	0	0	2	0	0	0	0	Group 2	66.01	67.91	66.96	50.65	60.26	55.46
M2	2	1	0	0	2	1	0	0							
M3	2	0	0	0	1	0	0	0							
M4	3	0	0	0	3	0	0	0	Group 3	69.39	62.51	65.95	63.69	60.21	61.95
F4	2	3	0	0	0	2	0	0							
F5	1	0	0	0	1	0	0	0	Group 4	62.14	68.05	65.10	52.20	49.30	50.75
F6	6	0	0	0	5	0	0	0							
F7	0	1	1	0	0	0	3	0	Group 5	61.82	69.38	65.60	60.69	61.42	61.06
M5	0	0	1	1	0	0	2	0							
	**N = 13 Without DAP				**N = 13 With DAP					N = 13 With DAP			N = 13 Without DAP		
Dog ID	**Frequency of Barking**	**Frequency of Paws on Fence**	**Frequency of Lying**	**Frequency of Low Posture**	**Frequency of Barking**	**Frequency of Paws on Fence**	**Frequency of Lying**	**Frequency of Low Posture**	**Group Number**	**dB Reading AM**	**dB Reading PM**	**dB Reading Average**	**dB Reading AM**	**dB Reading PM**	**dB Reading Average**
M6	0	0	0	0	0	0	0	0	Group 6	70.41	69.40	69.91	71.53	74.13	72.83
M7	0	0	0	0	0	0	1	0							
M8	2	2	0	0	2	0	0	0							
M9	1	0	0	0	2	0	0	0	Group 7	53.82	60.53	57.18	64.38	71.99	68.19
M10	0	0	0	0	0	0	0	0							
M11	7	2	0	0	7	2	0	0							
M12	0	0	1	0	0	0	0	0	Group 8	53.46	54.49	53.98	60.37	63.08	61.72
F8	6	0	0	0	6	0	0	0							
M13	0	0	0	0	0	0	0	1							
F9	0	0	2	0	0	0	3	0	Group 9	52.22	52.72	52.47	49.59	51.97	50.78
M14	0	0	0	0	1	0	0	0							
M15	2	0	0	0	3	0	0	0	Group 10	47.64	58.91	53.28	58.89	58.91	58.90
M16	2	0	0	0	3	0	0	0							

*dB readings were recorded in both the morning (AM) and afternoon (PM) and were recorded as an average reading per trial/per condition. ** Note: The conditions were counterbalanced (without DAP/with DAP, n = 12, with DAP/without DAP, n = 13).